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**Diminished coagulation capacity assessed by calibrated automated thrombography
during acute Puumala hantavirus infection**

Running head: Decreased endogenous thrombin potential in hantavirus infection

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Foundation.

Conflicts of interests: none.

24 **Abstract**

25 **Objectives**

26 Coagulation abnormalities are associated with Puumala virus-induced hemorrhagic fever with renal
27 syndrome (PUUV-HFRS). We evaluated the coagulation capacity of plasma during acute PUUV-HFRS by
28 measuring thrombin generation using calibrated automated thrombography (CAT®).

29 **Material and Methods**

30 The study cohort comprised of 27 prospectively collected, consecutive, hospital-treated patients with acute
31 PUUV infection. Blood samples were drawn in the acute phase and at the control visit approximately 5
32 weeks later. To evaluate thrombin generation, the lag time of initiation, endogenous thrombin potential
33 (ETP), and peak and time to peak thrombin concentration were assessed by CAT® in platelet poor plasma
34 without corn trypsin inhibitor. Plasma levels of D-dimer, fibrinogen and prothrombin fragments (F1+2) were
35 also evaluated.

36 **Results**

37 When the acute phase was compared with the control phase, ETP was decreased (median 1154 nM/min,
38 range 67-1785 vs. median 1385 nM/min, range 670-1970; $p<0.001$), while the lag time was prolonged
39 (median 3.8 minutes, range 2.1-7.7 vs. median 2.9 minutes, range 2.0-4.1; $p<0.001$). Low ETP correlated
40 with low peak thrombin concentration ($r=0.833$, $p<0.001$). Prolonged time to peak associated with the lag
41 time ($r=0.78$, $p<0.001$). ETP was associated with thrombocytopenia ($r=0.472$, $p=0.015$) and weakly with
42 fibrinogen level ($r=0.386$, $p=0.047$). The measured CAT® parameters did not associate with D-dimer and
43 F1+2 levels.

44 **Conclusions**

45 Decreased ETP together with low peak and prolonged lag time indicate decreased plasma potential for
46 thrombin generation *in vitro*. Together with low platelet count and enhanced fibrinolysis this further refers to
47 altered blood coagulation and increased propensity toward bleeding in acute PUUV-HFRS.

49 **Keywords:** coagulation; Calibrated automated thrombography; thrombin; hantavirus; platelet; fibrinolysis

50 **Abbreviations:** ADAMTS13, a thrombospondin type 1 domain; APTT, activated partial thromboplastin

51 time; AT, antithrombin; CAT®, calibrated automated thrombography; CRP, C-reactive protein; ETP,

52 endogenous thrombin potential; F1+2, prothrombin fragments; HCPS, hantavirus cardiopulmonary

53 syndrome; HFRS, hemorrhagic fever with renal syndrome; LT, lag time; PC, protein C; PPP, platelet poor

54 plasma; PS, protein S free antigen; PT, prothrombin time; PUUV, Puumala virus; TAFI, thrombin

55 activatable fibrinolysis inhibitor; TF, tissue factor; TG, thrombin generation; tPA, tissue plasminogen

56 activator; TT, thrombin time; tt Peak, time to peak.

57 **Introduction**

58 Hantaviruses are the cause of two disease entities, hemorrhagic fever with renal syndrome (HFRS) in Europe
59 and Asia, and hantavirus cardiopulmonary syndrome (HCPS) in North and South America. Puumala
60 hantavirus (PUUV) causes mild HFRS, also called nephropathia epidemica in Europe [1, 2]. PUUV-HFRS is
61 characterized by thrombocytopenia and coagulation abnormalities, acute kidney injury and capillary leakage
62 [1-3]. Petechiae, epistaxis, hematuria and conjunctival bleedings are common [1]. Hemorrhagic gastropathy
63 is observed in all PUUV-HFRS patients in gastroscopy [4]. Severe and fatal hemorrhages of pituitary gland,
64 kidneys, heart, liver, lungs and peritoneal cavity have been described [5, 6]. The risk for cardiovascular
65 disease has also been linked with PUUV-HFRS [7]. Disseminated intravascular coagulation has been
66 encountered in severe cases [8, 9].

67 Hantaviruses target vascular endothelial cells via $\beta 3$ integrin receptor and adhere quiescent platelets to the
68 endothelium, thus contributing to vascular permeability and thrombocytopenia [10]. Through interactions
69 with hantavirus, platelets and endothelium, alterations in the coagulation system occur. Previous studies
70 imply enhanced thrombin formation, as evaluated by shortened prothrombin time (PT) and thrombin time
71 (TT), and overall increase in prothrombin fragments 1+2 (F1+2), and decreased levels of natural
72 anticoagulants, antithrombin (AT), protein C (PC) and protein S free antigen (PS) [8, 11]. A study with
73 PUUV infected human umbilical vein endothelial cells suggests increased tissue factor (TF) activity [12].
74 Fibrinolysis is activated as indicated by increased concentrations of fibrin degradation products, D-dimer and
75 tissue plasminogen activator (tPA) [8, 13]. Platelet ligands are altered, and ADAMTS13 activity is decreased
76 [14].

77 Thrombin is the key enzyme during coagulation leading to the conversion of fibrinogen to fibrin and clot
78 formation. Thrombin generation (TG) assays are useful indicators of the overall plasma coagulability, in
79 contrast to the conventional coagulation tests that mainly assess individual factors or a part of the
80 coagulation pathway. Calibrated automated thrombography (CAT®) measures *in vitro* TG of plasma by
81 continuous cleavage of a fluorogenic substrate, thus expressing the overall haemostatic potential [15]. CAT®

is applied in research of vascular thrombosis, bleeding disorders and monitoring of anticoagulant treatment [16]. To our knowledge, studies on TG by CAT® in hantavirus infections are yet lacking.

Both hemorrhagic and thrombotic events have been associated with PUUV-HFRS, but the underlying mechanisms of alterations in coagulation system are not well defined. Therefore, we aimed to evaluate the plasma coagulation capacity in PUUV-infected patients by measuring TG by CAT®. The goal was to describe how TG is altered during the acute phase of infection, and further determine the possible hypo- or hypercoagulability associated with hantavirus infection. We also sought to investigate the possible associations of CAT® assay with the tests applied to measure thrombin formation and fibrinolysis in clinical practice and variables depicting disease severity of acute PUUV-HFRS.

Material and Methods

Ethics statement

All patients were recruited and enrolled after providing a written informed consent. The study protocol was approved by the Ethics Committee of Tampere University Hospital. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Patients

The study was carried out in Tampere University Hospital, University of Tampere, Helsinki University Hospital and University of Helsinki. All patients came from the Pirkanmaa area and were hospitalized at Tampere University Hospital due to serologically confirmed acute PUUV-HFRS [17] during the period from October 2010 to February 2014.

Twenty-seven prospectively collected, consecutive patients (17 males) with acute PUUV-HFRS were included. Their median age was 50 years (range 21-67 years). None of the subjects used anticoagulant or immunosuppressive therapy. Two patients used an anti-platelet drug (acetylsalicylic acid).

104 *Clinical and laboratory data*

105 The following variables were recorded: the number of days from the onset of fever before the acute-phase
106 study samples were collected, the length of hospital stay (days), signs of bleeding symptoms (yes/no),
107 thromboembolic complications (yes/no), need for transient hemodialysis treatment (yes/no), and maximum
108 gain in weight (kg). Complete blood count, plasma C-reactive protein (CRP) and plasma creatinine were
109 measured according to clinical need. The laboratory analyses were carried out at the Laboratory Centre of
110 Pirkanmaa Hospital District using standard methods.

111 *Methods*

112 The study design was longitudinal with two time-points of blood draw for CAT®. The acute phase samples
113 (n=27) were taken median 7 days (range 4-12 days) from the onset of fever. Control samples (n=23) were
114 taken at the follow-up visit, median 43 days (range 38-76 days) from the onset of fever. The blood count was
115 assessed in the acute and control phase of CAT® study days, and the lowest platelet count during the
116 hospital stay was recorded.

117 CAT® analyses and plasma measurements of fibrinogen, F1+2 and D-dimer were carried out in Clinical
118 Chemistry coagulation laboratory (HUSLAB Laboratory Services, Helsinki University Central Hospital,
119 Finland). D-dimer (Tina Quant D-Dimer®, Roche Diagnostics, Mannheim, Germany) and fibrinogen
120 (Multifibren U® Siemens Healthcare Diagnostics) levels were determined according to manufacturer's
121 recommendations. F1+2 were measured by an enzyme immunoassay (Enzygnost® F1+2, monoclonal,
122 Siemens Healthcare Diagnostics). The reference values for D-dimer were ≤ 0.5 mg/l, fibrinogen 1.7-4.0 g/l
123 and F1+2 69-229 pmol/l.

124 *Measurement of thrombin generation by CAT®*

125 TG was measured using CAT® (Diagnostica Stago) with the Stago PPP reagent (tissue factor 5 pM and
126 phospholipids 4 μ M) without corn-trypsin inhibitor. The lag time of the initiation of TG (LT, min), the
127 endogenous thrombin potential (ETP; the area under the curve; nM thrombin x time), peak (maximum
128 thrombin concentration, nM) and time to peak (tt Peak, min) were measured and recorded according to the

129 manufacturer's instructions. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mM)
130 tubes according to the local sampling protocol as part of hospital routine, and centrifuged (at 2500 g for 15
131 min). The PPP was separated within 2 hours and stored at -80 °C before analysis.

132 *Statistics*

133 All continuous, skewed variables were determined as medians and ranges. The associations between TG
134 markers and clinical and laboratory variables were evaluated for continuous data by Spearman rank
135 correlation coefficient. To analyze the changes between the acute and control phase, Wilcoxon-signed rank
136 test was used for pairwise comparisons. The level of significance was set at p value 0.05 (2-tailed). Statistical
137 analyses were performed with IBM SPSS Statistics for Windows version 22.0 (Armonk, NY, USA).

138 **Results**

139 *Clinical and laboratory findings*

140 All 27 patients suffered from clinically typical and serologically confirmed PUUV-HFRS [17]. The clinical
141 and laboratory findings of the patients are shown in Table 1. Mild bleedings were reported in eight patients
142 including nasal and conjunctival hemorrhages, petechiae, hemoptysis and melena. There were no
143 thromboembolic events recorded. None of the patients needed transient hemodialysis treatment.

144 The acute phase median platelet count was $68 \times 10^9/l$ (range $8-222 \times 10^9/l$), CRP 57 mg/ml (10-178 mg/ml),
145 hemoglobin 139 g/l (120-177 g/l), hematocrit 0.40 (0.34-0.49) and creatinine 126 $\mu\text{mol/l}$ (52-699 $\mu\text{mol/l}$).
146 Twenty-four out of 27 patients were thrombocytopenic (lowest platelet count $<150 \times 10^9/l$).

147 *Thrombin generation by CAT®*

148 When compared with the control phase, ETP was diminished by 16% (1154 nM/min, 67-1785 nM/min vs.
149 1385 nM/min, 670-1970 nM/min; $p<0.001$). Additionally, tt peak was prolonged (7.3 minutes, 4.8-14.9
150 minutes vs. 5.9 minutes, 4.3-9.8 minutes; $p=0.012$). Peak thrombin concentration was lowered (204 nM, 5.6-
151 293 nM vs. 243 nM, 106-331 nM; $p=0.008$), and LT was prolonged (3.8 minutes, 2.1-7.7 minutes vs. 2.9
152 minutes, 2.0-4.1 minutes; $p<0.001$) in the acute phase. Accordingly, ETP correlated with peak thrombin

153 concentration ($r=0.833$, $p<0.001$; Fig. 1A). Tt peak associated with the LT ($r=0.78$, $p<0.001$; Fig. 1B). An
154 inverse correlation was observed between peak and tt peak ($r= -0.54$, $p=0.004$).

155 *Associations of CAT® parameters with variables depicting clinical disease*

156 In the acute phase, ETP associated with the platelet count measured in the CAT® study day sample ($r=0.472$,
157 $p=0.015$; Fig. 1C), and with the lowest platelet count measured during the hospital stay ($r=0.402$, $p=0.038$).
158 Similarly, peak thrombin concentration associated with the platelet count of the CAT® study day ($r=0.554$,
159 $p=0.003$), and with the lowest platelet count measured during the hospital stay ($r=0.462$, $p=0.015$). Prolonged
160 LT and low ETP associated with increased fibrinogen level measured in the acute phase ($r=0.511$, $p=0.006$
161 and $r=0.386$, $p=0.047$, respectively; Fig. 1D). The fibrinogen level was acutely increased compared with the
162 control phase (median 4.2, range 2.2-9.6 g/l and median 3.4 g/l, range 2.6-4.9 g/l, respectively; $p=0.005$).

163 D-dimer was increased in the acute phase (2.8 mg/l, 0.6-34 mg/l vs. 0.4 mg/l, 0.2-1.1 mg/l; $p<0.001$).

164 D-dimer associated strongly with F1+2 ($r=0.843$, $p<0.001$). F1+2 was increased acutely (704 pmol/l, 284-
165 1875 pmol/l vs. 263 pmol/l, 118-556 pmol/l; $p<0.001$). ETP did not associate with D-dimer and F1+2
166 ($r=-0.079$, $p=0.695$ and $r=-0.164$, $p=0.415$, respectively). There were no associations between peak and D-
167 dimer and F1+2 ($r=-0.030$, $p=0.882$ and $r=-0.162$, $p=0.418$, respectively).

168 Clinical variables depicting the disease severity, i.e. maximum leukocyte count, maximum plasma creatinine,
169 CRP level and length of hospital stay were not associated with CAT® parameters (data not shown).

170 Furthermore, reduced ETP was not associated with bleedings (data not shown).

171 **Discussion**

172 The primary aim of this study was to investigate whether thrombin generation (TG), measured using
173 calibrated automated thrombography (CAT®) assay, is altered during acute Puumala virus -induced
174 hemorrhagic fever with renal syndrome (PUUV-HFRS). In addition, we sought to elucidate the underlying
175 coagulation mechanisms predisposing to bleeding and thrombosis. The main findings were reduced
176 endogenous thrombin potential (ETP) and peak thrombin concentration suggesting diminished plasma
177 potential for TG during acute PUUV-HFRS. Prolonged lag time (LT) indicating slower initiation of burst,

178 and extended time to reach the peak representing the velocity of TG, further support the finding of
179 hypocoagulability. Decreased platelet count, one of the clinical characteristics of hantavirus infection, was
180 found to associate with low ETP and low peak thrombin concentration. We did not find statistically
181 significant associations between prothrombin fragments F1+2 (F1+2), D-dimer and CAT® parameters in this
182 study population.

183 Previous studies imply enhanced TG in acute PUUV-HFRS on the basis of increased prothrombin fragments
184 F1+2 generated during conversion of prothrombin to thrombin, and an increased level of fibrin degradation
185 product D-dimer [8, 9, 11]. High levels of circulating F1+2 and D-dimer observed in this study confirm
186 previous findings. *In vivo* TG parameters, F1+2 and D-dimer, strongly depend on the amount of tissue factor
187 (TF) and thrombomodulin present on vascular endothelial cells [16, 18]. *In vivo* TG is also ongoing in the
188 microparticles released during infection [19, 20]. Data suggests increased TF in endothelial cells in the acute
189 phase of PUUV-HFRS [12]. On the other hand, the *in vitro* thrombin potential assessed by CAT®
190 determines how thrombin can be generated by plasma containing a defined amount of TG trigger. Thus, it
191 measures the haemostatic balance of plasma clotting factors and inhibitors independently of the procoagulant
192 and inhibitory drivers released by the endothelium [18]. The difference between *in vivo* and *in vitro* TG is
193 well reflected in consumption coagulopathy, a condition where indicators of ongoing coagulation are
194 increased, but plasma potential of TG is decreased [21].

195 A previous study indicates slightly prolonged prothrombin time (PT), prolonged activated partial
196 thromboplastin time (APTT), and shortened thrombin time (TT) during acute PUUV-HFRS [8, 18]. These *in*
197 *vitro* coagulation tests cannot detect the *in vivo* contribution of endothelial cells and shear stress of blood
198 flow on local clot formation and fibrinolysis. Traditional coagulation tests APTT and PT assess the time to
199 the initiation of clot formation and thus they do not entirely reflect the hemostatic balance in acutely ill
200 patients [22]. These plasma clotting assays are considered to reflect the LT phase of TG in CAT® assay [22].
201 Our observation of prolonged LT indicating slower initiation of TG is in line with the previous findings of
202 the coagulation tests APTT and PT. As the data concerning *in vitro* coagulation tests is lacking in the current
203 study population, the direct comparison with CAT® parameters is not possible.

204 Fibrinolysis is increased, as indicated by high D-dimer and endothelial cell tissue plasminogen activator
205 (tPA) levels, during PUUV infection [8, 11-13]. The plasminogen activator inhibitor 1 (PAI-1) level is not
206 altered in the acute phase [13]. Thrombomodulin-associated thrombin activates the thrombin activatable
207 fibrinolysis inhibitor (TAFI), which downregulates fibrinolysis. It can be speculated that low ETP may
208 contribute to decreased TAFI and thus increased fibrinolysis and bleeding tendency [23]. Diminished TG in
209 PUUV-HFRS together with excessive fibrinolysis also resembles the data obtained in another hemorrhagic
210 fever, dengue virus infection [24, 25]. In dengue fever the bleeding complications have been shown to
211 associate with reduced thrombin formation along with thrombocytopenia and enhanced fibrinolysis [26]. We
212 did not find an association between low ETP and bleedings, although mild bleedings were reported in one
213 third of the patients.

214 We are aware of the relatively small sample size of the study. Yet, the associations were statistically
215 significant, even if the number of clinical events remained minor. The levels of coagulation factors were not
216 available and individual acquired or inherited factors affecting hemostasis could not be assessed. Two
217 patients used aspirin, a platelet antagonist that inhibits platelet aggregation and thrombin formation. As TG
218 was assessed in platelet poor plasma it is unlikely that aspirin, attached to the minor amount of residual
219 platelets, could affect these CAT® results.

220 Ongoing *in vivo* coagulation may result in consumption of platelets and coagulation factors during acute
221 PUUV-infection. Correlation of low platelet count with low ETP and low peak may imply thrombin
222 activation and consumption of platelets, as thrombopoiesis is shown to be active during acute PUUV-HFRS
223 [20]. Natural anticoagulants, protein C and protein S free antigen and antithrombin are also found to be
224 decreased in the acute phase of PUUV-HFRS [8]. Increased TF expression on the endothelial cells and
225 microparticle release might result in consumption of platelets and clotting factors resulting in lower ETP. All
226 of these findings are supported by the previous reports [8, 11, 20].

227 In conclusion, in this study we found decreased *in vitro* TG measured by CAT® in acute PUUV infection.
228 Together with thrombocytopenia, increased fibrinolysis and signs of enhanced TG *in vivo* this data suggests
229 a mild to moderate consumption coagulopathy during acute PUUV-HFRS. The CAT® results of plasma

hypocoagulability support previous findings of impaired hemostasis during acute PUUV-HFRS. Larger future studies might further clarify the role of coagulation in the pathogenesis of HFRS.

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300 Figure Legend

301

302 Figure 1. Scatter plots illustrating the correlation between endogenous thrombin potential and peak thrombin
303 concentration (Fig.1A), lag time and time to peak thrombin concentration (Fig. 1B), endogenous thrombin
304 potential and simultaneous platelet count (Fig. 1C) and endogenous thrombin potential and plasma
305 fibrinogen level (Fig. 1D) during acute PUUV infection.

306

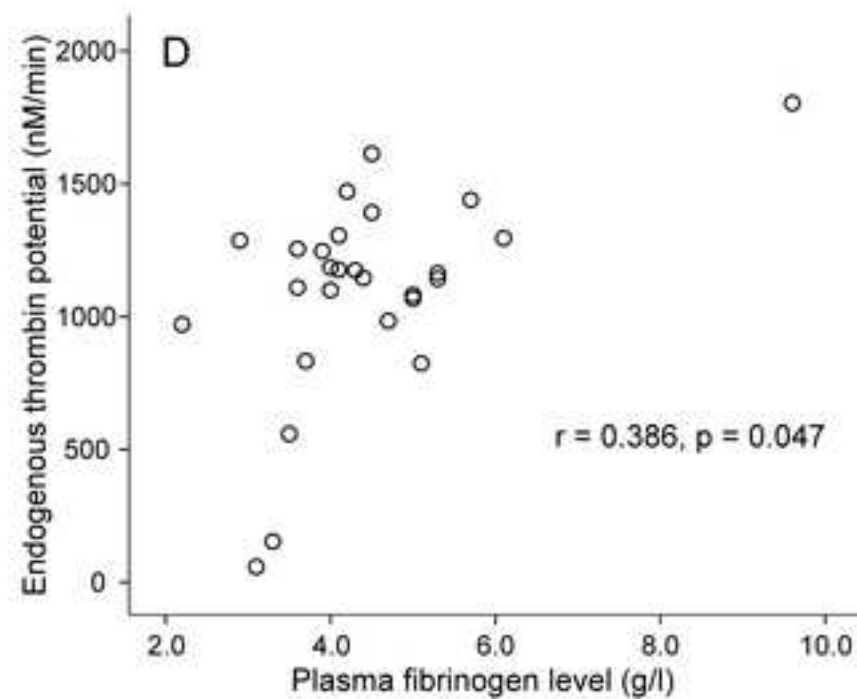
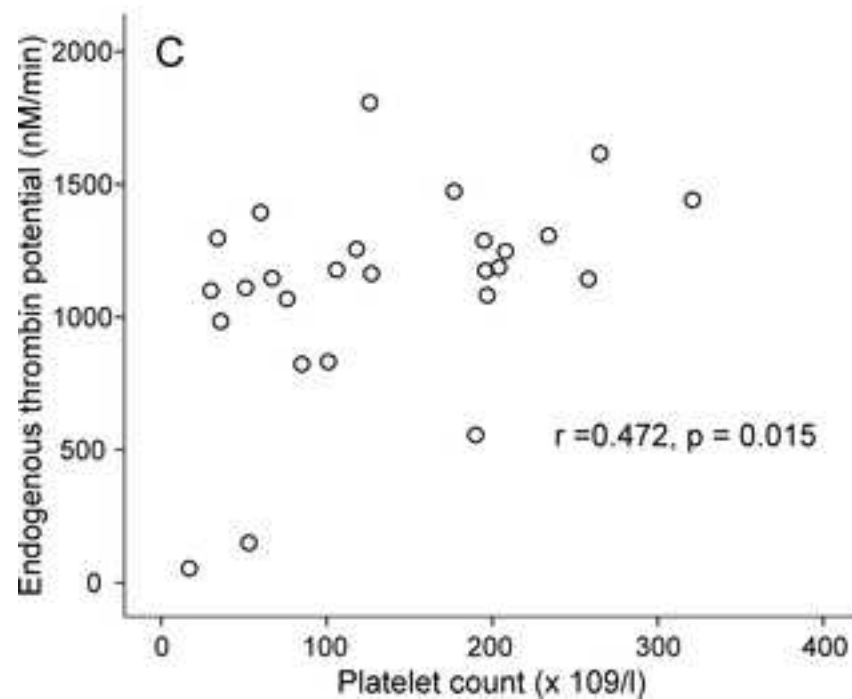
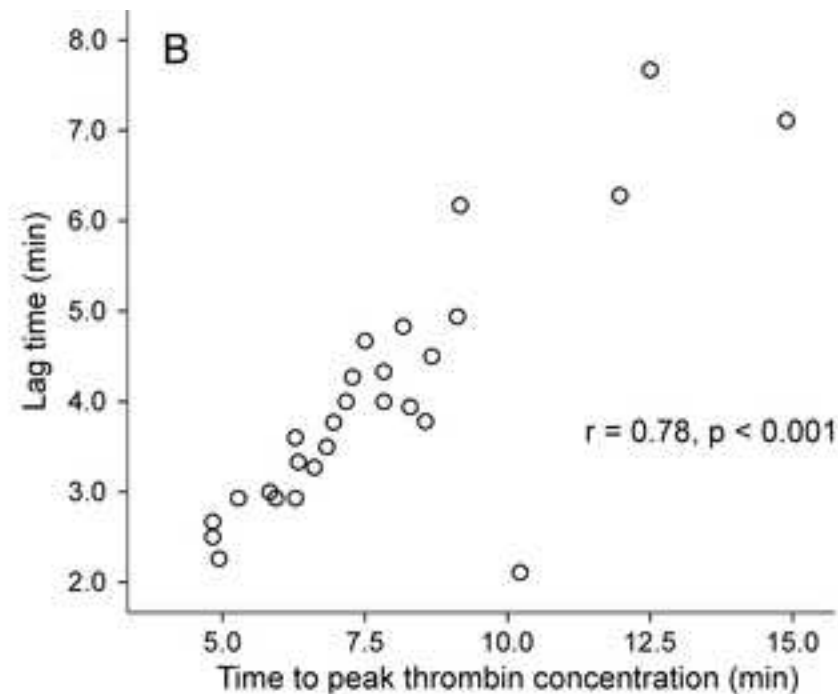
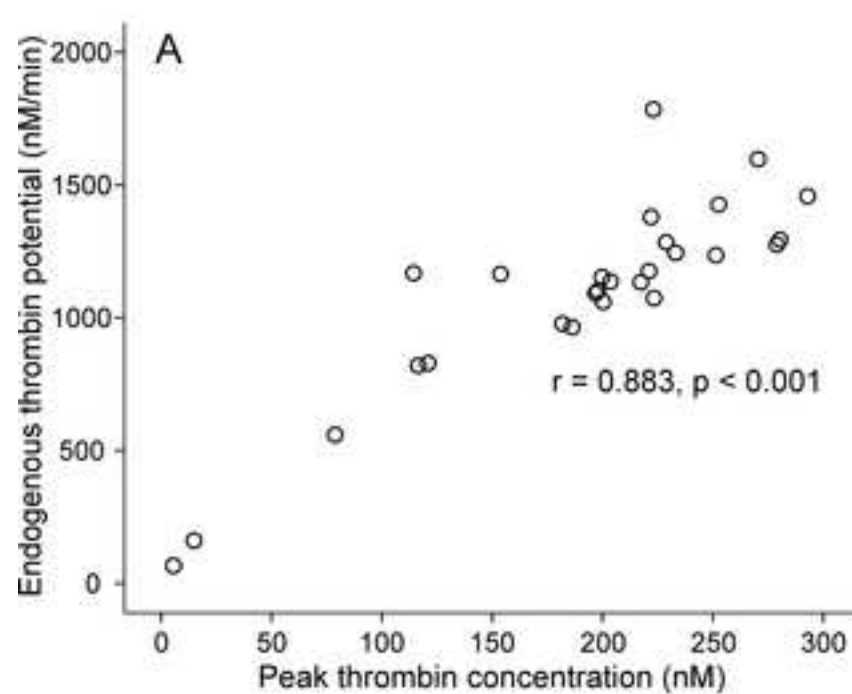


Table 1. The clinical and laboratory findings during hospital care in 27 patients with acute Puumala hantavirus infection.

Clinical or laboratory variable	Median	Range
Days from the onset of illness ^a	7	4-12
Length of hospital stay (days)	7	3-12
Body mass index (kg/m ²)	26.6	22.3-36.8
Change in weight (kg) ^b	3.8	0.5-11.3
Systolic BP min (mmHg)	108	80-135
Diastolic BP min (mmHg)	67	55-83
Creatinine max (μmol/l)	268	71-983
Leukocyte count max (x 10 ⁹ /l)	10.7	4-45
Hemoglobin max (g/l)	155	122-214
Hematocrit max	0.43	0.37-0.60
Platelet count min (x 10 ⁹ /l)	60	5-150
CRP max (mg/ml)	79	21-244

Abbreviations: min=minimum, max=maximum, BP=blood pressure.

Reference values: hematocrit 0.35-0.50 for men and 0.35-0.46 for women, platelet count 150-360, leukocyte count 3.4-8.2 x 10⁹/l, CRP < 10 mg/ml, creatinine < 105 μmol/l for men and < 95 μmol/l for women.

^aThe number of days of fever before the first study samples were obtained.

^bReflects fluid accumulation in the oliguric phase of PUUV-HFRS.